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Transcription factor 4 (TCF4) and schizophrenia: integrating the animal and the human perspective

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Transcription factor 4 (TCF4) and schizophrenia: Integrating the animal and the human perspective

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Abstract

Schizophrenia is a genetically complex disease considered to have a neurodevelopmental pathogenesis and defined by a broad spectrum of positive and negative symptoms as well as cognitive deficits. Recently, large genome-wide association studies have identified common alleles slightly increasing the risk for schizophrenia. Among the few schizophrenia-risk genes that have been consistently replicated is the basic Helix-Loop-Helix (bHLH) transcription factor 4 (*TCF4*). Haploinsufficiency of the *TCF4** gene causes the Pitt-Hopkins syndrome - a neurodevelopmental disease characterized by severe mental retardation. Accordingly, *Tcf4* null-mutant mice display developmental brain defects. *TCF4* associated risk alleles are located in putative coding and non-coding regions of the gene. Hence, already subtle changes at the level of gene expression might be relevant for the etiopathology of schizophrenia. Behavioural phenotypes obtained with a mouse model of slightly increased gene dosage and electrophysiological investigations with human risk-allele carriers revealed an overlapping spectrum of schizophrenia-relevant endophenotypes. Most prominently, early information processing and higher cognitive functions appear to be associated with *TCF4* risk genotypes. Moreover, a recent human study unravelled gene*environment interactions between *TCF4* risk alleles and smoking behaviour that were specifically associated with disrupted early information processing. Taken together, *TCF4* is considered as an integrator ('hub') of several bHLH networks controlling critical steps of various developmental and possibly also plasticity related transcriptional programs in the CNS and changes of *TCF4* expression appear to affect brain networks important for information processing. Consequently, these findings support the neurodevelopmental hypothesis of schizophrenia and provide a basis to identify the underlying molecular mechanisms.

Keywords

Schizophrenia, neurodevelopment, transcription factor 4, *TCF4*, *E2-2*, *SEF2*, *ITF2*, *ME2*, gating, cognition, mouse models, smoking, endophenotype, polymorphism, SNP, basic Helix-Loop-Helix protein

* Formatting is following IUPAC nomenclature:

TCF4 protein/ protein function

Tcf4 rodent gene cDNA mRNA

TCF4 human gene cDNA mRNA

Introduction

Schizophrenia is still an unsolved genetic enigma. Although the disease is clearly heritable and great effort has been undertaken in the past decades to elucidate the genetic basis of this disorder, no major risk genes that would be suitable for prediction of the illness have been identified. The reason for this failure might arise from complex multigenetic interactions of risk alleles with minor individual contributions. It is also likely that a disease entity 'schizophrenia' does not exist behind the phenomenology-based disease classifications, which were useful for the clinical requirements but not for biological research. Thus, in the following text schizophrenia-spectrum disorder is meant when schizophrenia is written due to an improved readability. Nevertheless, in the last years some interesting and replicable findings with population-wide significance suggest that variations in a few genes might serve as risk markers at least in a subgroup of schizophrenia patients. Among the most validated genes is the basic Helix-Loop-Helix (bHLH) transcription factor 4 (*TCF4*). The preclinical and clinical findings regarding the connection between the *TCF4* gene and schizophrenia will be reviewed and discussed in this contribution.

The basic-Helix-Loop-Helix protein TCF4

TCF4 belongs to the superfamily of bHLH transcription factors that can act as a transcriptional repressor or activator in a context specific fashion [1]. The bHLH domain comprises the basic region mediating DNA binding and a dimerization interface provided by the HLH domain with two amphipathic α -Helices separated by an unstructured loop region forming left-turned four-helix bundles in dimers [2, 3]. bHLH proteins are involved in various developmental processes, including control of proliferation, determination of cell fate and specifications but have also been shown to be transcriptional integrators of adaptive cellular processes in terminally differentiated cells [1, 4–6]. TCF4 (also known as E2-2/SEF2, ITF2, ME2) is an ubiquitously expressed protein and subgrouped with two additional so-called E-proteins, TCF3/E2A and TCF12/HEB, as class I bHLH factors [2](for complete lists of gene name assignments see e.g., <http://www.i-hop-net.org> or <http://www.ncbi.nlm.nih.gov/gene>). Ubiquitously expressed class I bHLH factors (TCF3, TCF4 and TCF12) are capable of forming homo-dimers and hetero-dimers with numerous cell-type specific (or

class II) bHLH and dominant-negative (or class V) HLH factors of the ID family (ID1-4) that lack a basic region and are therefore inhibiting DNA binding by sequestering bHLH factors [1].

Of particular practical importance is that the acronym or gene name alias *TCF4* (*TransCRIPTION Factor 4*) is unfortunately also widely used for T Cell Factor 4 (official gene symbol *TCF7L2*). *TCF7L2* belongs to the high mobility group (HMG) family of transcription factors and interacts with β -catenin of the WNT signaling pathway [7]. Therefore, greatest care should be taken when using software tools that automatically annotate key words from literature entries with '*TCF4*' and when manually scanning the '*TCF4*' literature. In consequence, the 'bHLH-*TCF4*-schizophrenia and Pitt-Hopkins Syndrome (PTHS)' related literature is likely to be contaminated by some false associations and authors, reviewers and readers should be sensible to this fact. To the best of our knowledge, we could not find evidence for a function of the bHLH factor *TCF4* in glia/oligodendrocyte development, which was unfortunately mentioned in several previous reviews.

Dimeric bHLH complexes bind to partially palindromic short DNA elements called Ephrussi-boxes (E-boxes) with the core sequence 5'-CANNTG-3' located in regulatory regions [8]. For structural reasons, individual bHLH proteins display a preference towards particular E-box half-sites, which however, does not necessarily predict the exact binding site of a given hetero-dimer that can even vary at different sites of the genome [9]. Class II bHLH factors cannot form homo-dimers and exert their transcriptional function only in concert with a class I or E-protein such as *TCF4* [1]. *TCF4* may thus exert pleiotropic functions depending on its dimerization partner(s) at a given developmental stage and in a particular cell type. In consequence, *TCF4* functions have been shown to be modulated by spatio-temporal expression patterns of its various interaction partners, differences in DNA-binding specificities, by post-translational modifications and associated co-factors [10–13].

Mammalian E-proteins have been shown to at least partially complement for each other and gene dosage effects have been described in lymphocyte development that further enhances the pleiotropic functions of these genes and complicates the assignment of dedicated roles for individual E-proteins [14, 15]. In contrast to mammals, only one E-protein is found in *Drosophila melanogaster* and *Caenorhabditis elegans*, i.e. daughterless (*da*) and helix-loop-helix protein 2 (*hlh-2*), respectively. Still, the corresponding mutants revealed multiple phenotypes including deficits in nervous system

development indicating phylogenetic conservation of E-protein functions [16–18]. The human and mouse genes coding for TCF4 are located on chromosome 18 in both species. The mouse gene *Tcf4* encompasses >360 kB (chr 18E2, forward strand) and the human *TCF4* >440 kB (chr 18q21.2, reverse strand)(www.ensembl.org, rel. 72). More than 18 coding splice variants with alternative N-termini have been described in humans [10] and the Ensembl genome browser (www.ensembl.org, rel. 72) lists 43 potentially protein encoding variants with the majority including the bHLH domain. Moreover, Sepp and colleagues [10] subgrouped 22 exons that are alternatively spliced particularly in the 5' region with multiple alternative transcription initiation sites. Up to date, two putative antisense and one micro RNA transcripts (miR4529) have been annotated on the opposite strand within the human *TCF4* locus (none so far in the mouse) potentially indicating regulation at the RNA level of increased complexity in the human genome. *TCF4* mRNA abundance levels and/or control of translation may be regulated by complementary micro RNAs (miRs) shown to bind to the 3' region of *TCF4* transcripts including the schizophrenia-associated risk factor miR-137 as well as miR-155 and miR-204 [19–22], the number of predicted and, however, so far experimentally not validated binding sites for additional miRs is much longer [23]. Besides the C-terminal located bHLH domain, TCF4 shares with the other class I/E-proteins additional regions of homology including two more N-terminally located transcriptional activation domains (AD1 and AD2) [24, 25]. These domains have been shown to provide protein-protein-interaction surfaces to recruit chromatin remodeling complexes and transcriptional co-factors such as CBP/p300 [26, 27]. Moreover, TCF4 may be part of a SWI/SNF chromatin-remodeling complex which might be of relevance for the etiopathology of schizophrenia [28]. A knockdown of endogenous *TCF4* in the human neuroblastoma cell line SH-SY5Y by siRNA altered the expression of multiple genes corresponding to various signalling pathways and affected cell survival, epithelial to mesenchymal transition and neuronal differentiation [29]. The siRNA approach yielded a highly efficient reduction of endogenous *TCF4* protein to levels below 20%. Therefore, these findings that were obtained in a proliferating neuroblastoma cell line could be of relevance for PTHS, where a loss-of-function of *TCF4* is most probable. Nonetheless, it is unclear, which bHLH interaction partners/dimeric complexes were affected in SH-SY5Y cells, e.g., affecting neuronal differentiation properties of this cell line and if these processes mimic developmental defects causing PTHS *in vivo*. Because of the presumably very subtle effects by the extragenic common alleles in *TCF4* that have

been associated with an increased risk of schizophrenia, mechanistic studies with relevance for schizophrenia are most likely technically more challenging.

In summary, the mammalian class I bHLH protein TCF4 can be considered as an integrator ('hub') of several bHLH networks controlling critical steps of various developmental and possibly also plasticity related transcriptional programs in the CNS (see Fig. 1). The deregulated splicing events and/or mRNA misexpression or altered stability of one or more distinct TCF4 protein isoform(s), which could be of particular relevance for schizophrenia are still unknown.

Schizophrenia

The main symptoms of schizophrenia can be distinguished into three major domains: 1) positive symptoms such as hallucinations, perceptual disturbances, delusional phenomena, and formal thought disorder; 2) negative symptoms mostly presented as flat affect, poverty of speech, avolition, anhedonia, lack of motivation, and inappropriate emotional responses; and 3) cognitive dysfunction including impairment of attention, memory, social cognition, and executive functions [30]. The highest risk period for developing schizophrenia is during young adulthood, while both sexes are equally affected by the disorder, although the age of onset is typically younger for men than women [31–33]. Although incidence rates vary depending on classification criteria, schizophrenia affects approximately 1% of the population across cultures [34, 35]. Individuals with parents or siblings suffering from schizophrenia have an increased risk for developing the disorder (8-12%). For monozygotic twins, the concordance rate is approximately 50% [36, 37]. The elevated familial incidence of schizophrenia strongly indicates that there must be a genetic contribution to the disorder, although the fact that concordance rates for monozygotic twins are lower than 100% suggests that environmental factors are also considerably involved. Thus, it is likely that a combination of genetic risk and environmental factors are required for the disorder to develop [37]. Initially, family-based linkage studies have identified several chromosomal regions and candidate genes that are associated with the risk for schizophrenia [38, 39]. However, none of the results of the linkage studies has passed a genome-wide significance level so far [40]. Subsequently, a multitude of association studies that were recently extended by genome-wide association studies (GWAS) identified only a few common

variants that contribute a very small increase in the susceptibility for schizophrenia [41–43]. Among the most replicable genes are the zinc finger binding protein 804A (*ZNF804A*), several genes from the major histocompatibility (MHC) region on chromosome 6, neurogranin (*NRGN*), and *TCF4* [44]. Most recently, also several rare submicroscopic chromosomal alterations – called copy number variants (CNV) – have been detected to cause schizophrenia or schizophrenia-like symptoms (e.g., as the case in 22q11-syndrome) [43, 44]. However, these rare chromosomal abnormalities cannot explain the pathogenesis of the majority of schizophrenia patients and are often also associated with physical abnormalities and mental retardation.

Although there is evidence for enlarged ventricles and decreased cerebral (cortical and hippocampal) volume associated with schizophrenia, there is not a distinct “diagnostic” neuropathology associated with the disease [38, 45, 46]. However, misplaced and clustered neurons, particularly in the entorhinal cortex, indicate problems of neuronal migration and suggest an early developmental anomaly [47–49]. Moreover, pyramidal neurons in the hippocampus and neocortex have been shown to have smaller cell bodies and fewer dendritic spines and dendritic arborizations and there are also reports of decreases in cell numbers in the thalamus and a decreased number of oligodendrocytes (reviewed in [39]. Additionally, decreased presynaptic proteins such as synaptophysin, SNAP-25, and complexin II have been observed in schizophrenia brains [50, 51] as well as decreased density of interneurons (e.g., parvalbumin-immunoreactive cells; [52, 53]. Neuroimaging data and post-mortem studies have shown that N-acetylaspartate (NAA), a marker of neuronal integrity, is decreased in first episode and never-medicated patients [54, 55]. Based on these neuropathological changes, investigators have conceptualized schizophrenia as a disease of functional “dysconnectivity” [56–58], or a “disorder of the synapse” [59, 60] affecting the machinery of the synapse and subsequent neurotransmission [50, 51].

Finally, accumulating evidence suggests that schizophrenia might be a neurodevelopmental disorder that is – at least in part – caused by aberrant early brain development that could be partially genetically determined: I.) Many schizophrenia patients exhibit delayed developmental milestones in childhood, including cognitive, motor, and behavioral abnormalities, which indicates abnormal brain function prior to diagnosis of schizophrenia, II.) obstetric complications and prenatal infections increase the risk for schizophrenia, III.) post-mortem studies did not find indicators for neurodegenerative

processes such as gliosis or loss of neurons in the brain of schizophrenia patients, and IV.) several anatomical and functional disruptions are associated with exacerbation of schizophrenia in adulthood and these disruptions can be simulated in developmental animal models [61, 62]. As suggested by Murray et al. [63], aberrant developmental processes may play a major role, especially in the congenital subform of schizophrenia that shows a gradual increase in behavioural disturbances until the disorder is diagnosed in adolescence or early adulthood. Maynard and colleagues [64] have proposed a two-hit hypothesis of schizophrenia. According to their suggestion a lesion occurring in early neurodevelopment (first hit), caused by genetic risk factors or adverse embryonic and perinatal events, in combination with a second hit, arising from hormonal events, excitotoxicity, psychosocial stress, or oxygen radical formation, may cause schizophrenia. Immunocytochemical and ultrastructural post-mortem studies have demonstrated neuronal alterations in schizophrenia, such as decreased size of the neuronal cell body, increased cellular packing density, fewer dendritic spines and synapses, and distortions in neuronal orientation [65]. The abnormalities in the cytoarchitecture, such as neuronal disarray, heterotopias, and malpositioning, indicate disruption of proliferation or migration at the gestational period [62]. In accordance, it was consistently shown that the expression of reelin, a glycoprotein that regulates neuronal migration, is strongly decreased in schizophrenia patients [66, 67]. Thus, these morphological and cytoarchitectural changes are likely to arise during brain maturation. In sum, several lines of evidence suggest that abnormalities in brain development may contribute to the pathogenesis of schizophrenia at least in a subset of patients.

Genetic association of TCF4 with schizophrenia

Since 14 years chromosome 18 has been repeatedly proposed as a possible location for schizophrenia and bipolar disorder risk genes [68–72]. As bipolar disorder and schizophrenia show a high genetic correlation [73] it is not surprising that *TCF4*, which is located on this chromosome, was initially associated with bipolar disorder: The first study found that bipolar disorder was associated with a CTG triplet repeat expansion in an intronic region of the *TCF4* gene [74]. The second study demonstrated that moderate expression of such repeats in this region was linked to severity of bipolar I disorder [75]. Subsequently, Pickard and colleagues [72] identified a pericentric inversion of chromosome 18 in a

small Scottish family whose members are suffering from mental retardation and schizophrenia and the breakpoint of this inversion was located close to the *TCF4* gene. More recently, several large but also partially overlapping meta-analyses of GWAS consistently identified that common variants of the *TCF4* gene contribute to the risk of schizophrenia (see also **Table 1**) [19, 76, 77]. In these analyses, two single nucleotide polymorphisms (SNPs) located in the intron located between the internal exon 4 and internal exon 5 of human *TCF4* gene according to the gene structure of Sepp et al., 2011 [10] (see below) on chromosome 18q21.2 (rs9960767, rs17512836) and an intragenic SNP near the *TCF4* gene (rs4309482) have shown the strongest association with the disease [19, 76, 77]. All three GWAS meta-analyses included data from the SGENE-plus study of schizophrenia, from the International Schizophrenia Consortium (ISC) and from the Molecular Genetics of Schizophrenia (MGS) group but the later reports of Steinberg et al. [77] and Ripke et al. [19] also included additional patient and control samples that are not overlapping. Additionally, three following studies replicated schizophrenia-*TCF4* gene associations in independent samples: (1) A study in Han Chinese (in which the rs9960767 SNP is not polymorphic) identified a further intronic *TCF4* SNP (rs2958182) that showed a significant association with schizophrenia [78]. (2) In a discovery sample from Ireland and a replication sample including non-overlapping samples from the Psychiatric GWAS Consortium (PGC), the SGENE-plus consortium and the Wellcome Trust Case Control Consortium 2 (WTCCC2), two intronic *TCF4* SNPs (again rs9960767 and rs17594526, which was among the top ten significant *TCF4* SNPs in the so far largest megalanalysis of Ripke et al. [19] passed the significance threshold of $p < 5 \times 10^{-8}$ [79]. (3) In a recent family-based linkage meta-analysis a further *TCF4* SNP was identified (rs1261117) to be significantly associated with schizophrenia [80]. The use of the family-based approach is a critical advantage here, given that all other GWAS employed only case-control designs that are susceptible for artefacts produced by population stratification [81], while using nuclear families in a replication study is robust against population stratification-induced false-positive findings [80].

Moreover, in a phenotype-based association study applied to the German GRAS (Göttingen Research Association for Schizophrenia) sample, *TCF4* rs9960767 (but not rs4309482) displayed some signal regarding a multivariate schizophrenia phenotype including PANSS positive and negative scores, a cognitive score, neurological soft signs, and age of prodromal onset [82]. Although the direction of the

effect was similar to previous GWAS (risk allele C was associated with a more pronounced phenotype) the association was not strong enough to pass multiple testing adjustments. In addition, a small post-mortem study suggested that at least the rs9960767 SNP is neither functional nor affects mRNA expression in the adult human brain indicating that such polymorphisms may yield their effects on gene expression through post-transcriptional pathways or in a developmental context by gene*environment interactions [41, 42]. In contrast, a more recent study reported that *TCF4* expression level in peripheral blood was significantly increased in patients with schizophrenia and bipolar disorder compared to controls. Additionally, peripheral *TCF4* mRNA concentration was positively correlated with severity of positive and negative symptoms. However, *TCF4* expression levels were only nominal and non-significantly correlated with some *TCF4* SNPs that have not been named as schizophrenia risk variants so far [83]. In the same study, after correction for multiple testing more than 10 *TCF4* SNPs, which have not been identified in previous GWAS, were significantly associated with expression of negative symptoms [83].

It was also investigated whether the *TCF4* polymorphism rs9960767 modulates the response to antipsychotic drug treatment in schizophrenia but in two independent samples, comprising of more than 200 patients in total, the clinical improvement across four weeks was not influenced by *TCF4* genotype [84] suggesting that this *TCF4* SNP is probably not a suitable predictor for antipsychotic drug effects.

Taken these findings together, SNPs from the *TCF4* gene together with common variants in the major histocompatibility complex (MHC) region are currently the best replicated schizophrenia susceptibility genes. However, the odds ratios for single variants are still small [OR around 1.2, see **Table 1**] and not useful for prediction of the disorder. Moreover, *TCF4* SNPs cannot predict antipsychotic drug response so far. Thus, either *TCF4* as well as the other schizophrenia risk genes only contribute a very small fraction to the total risk, together with many other genetic and environmental risks, or there is a distinct subpopulation of patients for which the *TCF4* abnormalities might be major contributors to the etiopathogenesis of schizophrenia.

***TCF4*, information processing, and cognition: Human studies**

Already Kraepelin (1909) and Bleuler (1911) proposed that attentional and information processing deficits constitute core symptoms of schizophrenia [85, 86]. Following the early idea of Arvid Carlsson that schizophrenia might be a “thalamic filter deficit disorder” [87], impairments in early information processing have been repeatedly suggested to play a critical role in the pathogenesis of schizophrenia [88–91]. Consequently, electrophysiological measures of early information processing – such as sensory gating or sensorimotor gating – have been proposed as promising behavioural endophenotypes of schizophrenia [92]. Such gating mechanisms have been conceptualized as important pre-attentive filter functions protecting cognitive processes from interfering with irrelevant information [93]. Schizophrenia patients and to a lesser extent also their unaffected first-degree relatives consistently display disrupted sensory and sensorimotor gating, commonly demonstrated by either lower P50 suppression of the auditory evoked potential (AEP) or reduced prepulse inhibition (PPI) of the acoustic startle response [88, 89, 94–100]. Both measures have been shown to be heritable and to be disturbed before onset of the illness [101–107]. Although sensory (P50 suppression) and sensorimotor (PPI) gating are conceptually related and both were parallel suggested as useful endophenotypes of schizophrenia they are not equivalent and usually also not correlated [94, 108–110]. However, a recent meta-analysis confirmed that electrophysiological gating measures differentiate best between healthy individuals, relatives of schizophrenia patients and the patients themselves when compared to other proposed endophenotypes such as ventricle size, neurological soft signs or neuropsychological dysfunction [111].

As described above, transgenic mice moderately overexpressing *Tcf4* in the postnatal brain display profound reductions in sensorimotor gating as measured by PPI [112]. Accordingly, the impact of the schizophrenia risk SNP *TCF4* rs9960767 on PPI was investigated in human samples (**Table 1**). In fact, the risk allele C of this SNP was strongly associated with reduced sensorimotor gating in two independent samples of healthy volunteers and schizophrenia patients [113]. Interestingly, low PPI levels (>1.5 SD below normal) have shown a much stronger associations (OR=4.81) with the *TCF4* risk allele C than schizophrenia per se (OR=1.23) [76]. When considering effect size measures, a similar pattern arises: whereas the association of a diagnosis of schizophrenia with *TCF4* genotype displayed only a very small effect size of $w=0.09$ [76], the association of the schizophrenia endophenotype PPI with *TCF4* showed a strong effect size of $d=0.90$ averaged across both samples.

Impressively, of the 23 subjects carrying the C-allele across both investigated samples, 14 (61%) displayed low PPI levels (>1.5 SD below normal), when compared to the merged total sample, which is again an expression of the strong genotype effect of *TCF4* on PPI. The authors hypothesized that the impact on PPI might arise from developmental changes of brain stem nuclei induced by the *TCF4* polymorphism (see Fig. 3 for an illustration of involved brain structures) [113].

Subsequently, we also investigated the influence of 21 *TCF4* polymorphisms – which were most strongly associated with schizophrenia in a recent meta-analysis [19] – on sensory gating as assessed by P50 suppression of the AEP [114]. We used a multi-centre study done including six academic institutions throughout Germany with 1821 subjects (1023 never-smokers, 798 smokers) randomly selected from the general population (**Table 1**). Given that smoking is highly prevalent in schizophrenia [115] and was shown to affect sensory and sensorimotor gating [116] several parameters for smoking behaviour were additionally assessed. Like PPI also P50 suppression was significantly decreased in carriers of schizophrenia risk alleles of the *TCF4* polymorphisms rs9960767, rs10401120, rs17597926, and 17512836 – the latter two were the most significant SNP in the mega-analysis of Ripke et al. [19]. Importantly, these gene effects were strongly modulated by smoking behaviour as indicated by significant interactions of *TCF4* genotype and smoking status: heavy smokers (Fagerström score ≥ 4) showed stronger gene effects on P50 suppression than light smokers and never-smokers. Moreover, the genotype*smoking interaction seems to be dose-related as the *TCF4* genotype effect grows with increasing smoking severity. Interestingly, *TCF4* genotype effects on sensory gating were more evident at frontal (Fz) than vertex (Cz) electrodes. Previous studies reported that the prefrontal cortex substantially contributes either to the sensory gating process *per se* [117] or at least to the generation of the P50 amplitude [118]. Additionally, data from a recent EEG source localization study suggest that the sensory gating deficit of schizophrenia patients could be explained by dysfunction of the dorsolateral prefrontal cortex [41]. Thus, *TCF4* mutations (in combination with smoking) might affect PFC function in schizophrenia. Accordingly, deficits of PFC functions have recently also been described in *Tcf4tg* mice [119].

In conclusion, these results imply that the schizophrenia risk alleles of *TCF4* variants interact with smoking behaviour with regard to auditory sensory gating. However, if smoking behaviour strongly modulates the *TCF4* genotype effects on a proposed endophenotype of schizophrenia it might also

modulate the risk for schizophrenia itself. We therefore suggested the investigation of potential moderating effects of dimensional and binary measures of smoking behaviour on genetic risk factors of schizophrenia. In fact, preliminary data from 882 schizophrenia patients and 2163 controls now suggest that the risk allele C of the *TCF4* rs9960767 is indeed more frequent in smoking schizophrenia patients (8.3%) than in non-smoking patients (5.3%) or smoking (5.5%) and non-smoking controls (5.8%) transferring in an OR of 1.55 for smoking patients in contrast to OR of 0.90 for non-smoking patients (personal communication Dan Rujescu, University of Munich, Germany, unpublished data). These results have certainly to be replicated in further and larger samples but nevertheless these data indicated that stratification for smoking behaviour in case-control association studies potentially adds power resulting in stronger gene effects. Moreover, it should be further explored if nicotine use itself might enhance the risk for schizophrenia as indicated by longitudinal studies showing that beyond cannabis and alcohol use also early consumption of tobacco increases the risk for psychosis [120, 121]. Finally, an extended endophenotype including electrophysiological gating measures such as PPI or P50 suppression, smoking behaviour, and risk genes such as *TCF4* may be suitable as an early indicator for a developing psychosis [114]. Moreover, dedicated gene*environment studies could be performed in mouse models providing additional evidence for *TCF4**smoking interactions and allowing the investigation of underlying molecular mechanisms.

Neurocognitive dysfunctions have been proposed as promising endophenotypes of schizophrenia as well [122]. In particular, impaired verbal memory, which is among the most prominent and consistently reported cognitive deficits of schizophrenia [123], has been emphasized as a potential intermediate schizophrenia phenotype as studies with unaffected relatives from multiple affected families (“multiplex families”) and twin studies demonstrated an increasing memory deficit along with an increasing genetic load [124–126]. Lennertz et al [127] therefore investigated the impact of the *TCF4* rs9960767 variant on verbal memory performance in a sample of 401 schizophrenia patients [127]. While no effect of the schizophrenia risk allele C on immediate recall and total learning was found, a weak trend regarding delayed verbal memory appeared surprisingly indicating superior performance in carriers of the risk allele compared to non-carriers. Moreover, in the cued recall condition (word recognition) schizophrenia patients carrying at least one C-allele significantly also

recognized more words compared to patients without the risk allele. These results were unexpected considering the supposed impact of *TCF4* on brain development and assuming that an endophenotype should display a similar association with the risk gene (e.g., impaired memory in carriers with the schizophrenia risk gene) as an endophenotype with the complex disease phenotype (e.g., memory deficit in schizophrenia patients). Given that the effects sizes of the genotype effects were rather small (Cohen's $d=0.34$, after correction for several covariates $d=0.27$) and that the results would not have become significant if the statistical threshold would have been corrected for multiple test parameters, these results should not be over-interpreted. These authors also explored functional effects of the same *TCF4* variant on a comprehensive neuropsychological test battery in a sample of about 200 schizophrenia patients and a control sample of 205 healthy volunteers [127]. The assessed cognitive functions attention and vigilance, working memory, processing speed, visuo-motor speed and set-shifting, as well as verbal fluency were all unaffected by *TCF4* rs9960767 in both groups (unpublished data). Thus, although haploinsufficiency of the *TCF4* gene is associated with severely disrupted intellectual functions as presented in the PTHS, no considerable effect of the *TCF4* rs9960767 polymorphism on neuropsychological function was found in this sample with exception of a weak and unexpected association with word recognition (Table 1).

In contrast, Wirgenes et al. recently reported from a large sample of patients with schizophrenia spectrum disorders (total $n=596$ including patients with schizophrenia, bipolar disorder, other psychoses) and healthy controls ($n=385$) that the risk alleles of the *TCF4* risk variants rs12966547 and rs4309482 were associated with worse verbal fluency in the total sample [83]. They found also some trends that the schizophrenia risk alleles from rs4309482 and rs9960767 were associated with ventricular and/or hippocampal volume but these results did not survive correction for multiple testing. In the exploratory analyses, there were also some significant associations of other *TCF4* SNPs with verbal learning, executive functioning, and several brain abnormalities [83].

A study in Han Chinese investigated the impact *TCF4* rs2958182 SNP, previously associated with schizophrenia in the same ethnicity [78], on cognitive functions in 580 schizophrenia patients and 498 controls [128]. The authors reported that the schizophrenia risk allele was associated with better performance in patients but worse performance in controls regarding an IQ test as well as in attention-related tasks. Because of this unexpected result pattern, the authors speculated that *TCF4* and

cognition might follow an inverted U-shaped function. However, it is not fully clear at the moment, if previous European studies and this Chinese study can be adequately compared.

Most recently, a Canadian study explored the association of the *TCF4* rs9960767 SNP with neurocognitive function in 173 first-episode psychosis patients (affective and non-affective psychosis). The authors reported that carriers of the rs9960767 C allele performed worse in the cognitive domains of “reasoning and problem solving” and “speed of processing” adumbrating that *TCF4* polymorphisms might contribute also to deficits in higher cognitive function in schizophrenia patients [129]

In a lymphocyte-based gene expression study in healthy Mexican Americans, it has been recently shown that peripheral expression of *TCF4* – among seven further genes (*IGFBP3*, *LRRN3*, *CRIP2*, *SCD*, *IDS*, *GATA3*, and *HNI*) – predicted cortical grey matter thickness measured with magnet resonance tomography [130]. Notably, *TCF4* expression was correlated particularly with grey matter thickness in the prefrontal cortex. The authors concluded that a progressive decline in the regenerative capacity of the brain contributes to normal cerebral aging including thinning of the grey matter [130]. A critical role of *TCF4* specifically for development of the prefrontal cortex was also supported by recent post-mortem data showing a significant association between *TCF4* expression and *cis* eSNPs (previously identified in an expression quantitative trait loci analysis) in tissue of the prefrontal cortex (rs1261085, rs1261134, rs1261073) and the thalamus (rs1261134), while in the hippocampus, temporal cortex and cerebellum no such associations were found [131].

Taken the human gating and cognition data together, it appears that *TCF4* SNPs likely affect early information processing in that way that schizophrenia risk alleles are consistently associated with a schizophrenia-like phenotype, i.e. reduced gating functions (for an overview see **Table 1**). At least the effect for auditory sensory gating was strongly modulated by smoking suggesting a possible gene*environment interaction that might be also relevant for the development of schizophrenia. There are also initial data arguing that common *TCF4* variants might have an impact on brain morphology specifically regarding the prefrontal cortex, which is also in line with the impact on gating functions that might involve the prefrontal cortex and also in accordance with neurodevelopmental phenotypes obtained with *Tcf4* and neuronal bHLH mouse models as discussed above. Whether common *TCF4* variants also influence higher cognitive functions in healthy volunteers or schizophrenia patients is not

clear at the moment as existing studies are controversial, present rather weak associations, and are currently not replicated. In contrast, more severe *TCF4* mutations, as occurring in the PTHS, are definitely accompanied with strong cognitive dysfunction suggesting that a considerably disturbed *TCF4* function is associated with strong changes in brain development (see following section).

TCF4 and neurodevelopmental disorders

Heterozygous hypomorphic, null mutations or deletion (haploinsufficiency) of the *TCF4* gene in humans causes the rare PTHS – an autosomal-dominant neurodevelopmental disorder characterized by severe mental, motor and language retardation, epilepsy, facial dysmorphisms, intermittent hyperventilation, and rarely also postnatal microcephaly –, pointing to the fact that *TCF4* is critical also for normal development of the mammalian nervous system (Forrest et al., 2012; de Pontual et al., 2009; Sweatt, 2013; Zweier et al., 2007). Currently, only 200-300 diagnosed cases with PTHS exists worldwide (Sweatt, 2013). A small proportion of patients suspected to have the Angelman syndrome, which displays a similar phenotype as PTHS and also have mutations in the *TCF4* gene [136]. A recent study with 10 young PTHS patients revealed strong intellectual and motor disabilities together with a behavioral phenotype that overlaps with autism spectrum disorders [137]. The autism-like behaviour was characterized by difficulties in engaging and communicating with others, frequent occurrence of repetitive motor stereotypies, repetitive play and fascination with specific objects, and difficulties with changes in daily life routines. The real age of the PTHS patients ranged from 32 to 289 months whereas the estimated developmental age lied between 3.5 months and 15 months for the mental abilities and between 4 months and 19 months for the motor abilities [137]. Notably, in a recent study investigating balanced chromosomal abnormalities in patients with autism convergent genomic information suggested that the *TCF4* gene might also be involved in the pathogenesis of autism-spectrum neurodevelopmental disorders [138].

Surprisingly, prominent macroscopic brain abnormalities are not common in PTHS: only subtle hypoplasia of the corpus callosum has been consistently reported [139–141], while also enlarged ventricles (similar to schizophrenia, [142] and thin hindbrain [140, 141] as well as enlarged caudate nuclei and a lower hippocampus volume have been reported [143]. Whalen et al estimated that only

about 50% of the PTHS patients display abnormalities in structural brain imaging, while only about 7% reveal a microcephaly Whalen et al (2012) .

***Tcf4/TCF4* expression in brain development**

The function of TCF4 in nervous system development and adult brain must be seen in the context of its numerous proposed and few experimentally validated interaction partners of the bHLH family (see Fig. 1) and its complex spatio-temporally regulated expression pattern. All E-proteins are expressed during embryonic stages including neural structures (<http://www.brain-map.org/>) with *Tcf4/TCF4* showing the highest expression levels in mouse and human brain tissue (<http://www.brainspan.org/>) [13, 144]. In contrast to *Tcf3* and *Tcf12*, *Tcf4* expression remains at substantially high levels in the adult and aged rodent brain [112, 145, 146]. *Tcf4* expression is sustained particularly in areas of high neuronal plasticity such as the cerebral cortex, hippocampus and cerebellum [13, 112]. Human *TCF4* expression has been detected in the prosencephalon and the ventricular zone of the embryonic CNS [133], in the telencephalon at all stages of fetal development as well as in the adult forebrain (<http://www.brainspan.org/>). In summary, *TCF4* is the only E-protein being expressed at all stages in the developing and adult mouse and human brain.

In contrast to the constitutive and broad expression of *Tcf4*, all putative interaction partners in the nervous system show a much more spatio-temporally restricted expression profile (Fig. 1). The expression of the bHLH inhibitors *Hes1* and *Id1* are transiently expressed in embryonal stages and their function indeed seems to be confined to inhibit premature differentiation initiation [147, 148]. Neurogenesis associated pro-neuronal class II bHLH proteins of the achaete scute (e.g., ASCL1/MASH1), atonal (e.g., ATOH1/MATH1) and neurogenin families (NEUROG1,2) are transiently expressed at early stages of development whereas the gradual onset of expression of group II bHLH proteins involved in terminal neuronal differentiation (e.g., NEUROD family members NEUROD1, -2, and -6) is confined to later stages and remains sustained in the adult brain [5, 149]. All type II neuronal bHLH proteins are thought to depend on the hetero-dimerization with an E-protein [1]. Thus, at least in later stages of neuronal differentiation and selected brain regions, TCF4 appears to be the obligate interaction partner of neuronal class II bHLH proteins. This selective availability as

unique interaction partner may explain dosage susceptibility of TCF4 observed in genetic model systems such as zebrafish [150] and mouse [151] and in human patients suffering from PTHS (Sweatt, 2013) and potentially also schizophrenia (see below).

***Tcf4* functions in neurodevelopment and cognitive processing: lessons from mouse models**

Tcf4 heterozygous null mutant mice (*Tcf4*^{+/-}) are viable, fertile and display no obvious phenotype [151]. Although subtle defects cannot be excluded so far, *Tcf4*^{+/-} mice do not replicate the profound effects observed in humans where haploinsufficiency causes severe developmental disturbances including PTHS phenotype (Fig. 2 and see below). As reported by Zhuang et al. [15, 152], *Tcf4* homozygous null mutant mice (*Tcf4*^{-/-}) were born with extremely low frequency and did not survive longer than one week after birth. In contrast, Flora et al. [151] did not observe embryonic lethality of null mutants and obtained expected Mendelian ratios of *Tcf4*^{-/-} mice at birth; however, animals died within the first 24h. Nevertheless, both studies showed that the complete inactivation of both *Tcf4* alleles has strong developmental consequences in mice with evident morphological defects detected so far only in pontine nuclei development, which has been specifically attributed to the interaction of TCF4 with the proneural transcription factor ATOH1/MATH1 [151]. Therefore, in mice TCF4 function during early brain development may be partially compensated by the other class I bHLH factors TCF3 and TCF12. However, in mosaic *Tcf4*^{+/-} *Tcf4*^{-/-} mice only pups displaying maximal proportion of 30% of *Tcf4* null cells are viable [153]. Nonetheless, conditional knockouts enabling targeted deletions at various stages of CNS development will be essential to better understand more subtle phenotypes possibly caused by the loss of function of *Tcf4*. Such mouse models may allow studying embryonal TCF4 dysfunction even in the adult brain without being hampered by embryonic or perinatal lethality.

So far, insight about the role of TCF4 on adult brain function in the mouse is restricted to a model with slightly increased expression levels in the forebrain [112]. In addition to loss-of-function models (see above), gain-of-function studies may be of particular relevance for schizophrenia as *TCF4* mRNA expression is significantly increased in post-mortem cortical samples and peripheral blood cells of

psychosis patients [83, 154]. Furthermore, *TCF4* mRNA expression level is elevated in neurons derived from human induced pluripotent stem cells of schizophrenia patients versus unaffected subjects [155]. Therefore, *Thy-1* promoter driven overexpression of *Tcf4* mRNA in brain structures involved in cognition such as the cortex and hippocampus of the mouse [112] may partially replicate molecular alterations of increased schizophrenia risk in humans. Subsequently, we will refer to these mice as *Tcf4tg*. The onset of transgenic *Tcf4* expression is confined to early postnatal stages and neither breeding problems nor any overt abnormalities have been observed. Nonetheless, adult *Tcf4tg* mice displayed profound deficits in contextual and cued delay fear conditioning indicating hippocampal deficits. Alterations in activity, anxiety or exploratory drive were not observed, thus postnatal *Tcf4* overexpression affected only early information processing and cognitive functions [112]. Fear associated learning deficits were erased upon applying stronger aversive stimuli arguing for a subtle defect [112]. In addition, *Tcf4tg* mice display deficits in trace fear memory most likely paralleled by reduced levels of attention and behavioural anticipation [119]. It has been shown that these higher order cognitive processes depend both on the hippocampus and the anterior cingulate cortex (ACC) [156]. Thus, impaired interactions between the prefrontal cortex and the hippocampus likely contribute to the reduced cognitive performance in *Tcf4tg* mice. Similar disturbances between remote brain regions have been described in the Df(16)A+/- strain that harbor a microdeletion in mice corresponding to a human chromosome 22 (22q11.2) deletion described in schizophrenia [157]. Moreover, altered functional cortical-hippocampal connectivity has been frequently reported in schizophrenia patients [158, 159]. In addition, *Tcf4tg* mice display sensorimotor gating deficits correlating with a frequent endophenotype of SZ patients [88, 107, 160–162]. In summary, the analysis of *Tcf4tg* mice has provided accumulating evidence to support the role of TCF4 in brain circuits involved in cognition and higher order information processing, which is independently strengthened by human studies (Fig. 3 and see below).

Discussion

The schizophrenia associated gene *TCF4* belongs to a subfamily of bHLH transcriptional factors that recognize E-box binding sites on regulatory DNA elements in the genome [1, 8]. At early developmental stages class I / E-protein transcription factors such as *Tcf3*, *Tcf4*, and *Tcf12* show wide expression throughout the brain but only *Tcf4* displays sustained expression in the adult brain of mice, which is most prominent in the cerebellum, hippocampus, and cortex [112, 145]. In conclusion, TCF4 is at least during later stages of neurodevelopment and in the adult brain the obligate interaction partner of multiple class II neuronal bHLH factors of e.g., the NEUROD family [112]. Therefore, TCF4 must be considered as an interaction ‘hub’ in neuronal bHLH protein networks important for different aspects of neurodevelopment and adult plasticity [149, 163]. Due to potentially competing functions, it is retrospectively not surprising that control of *TCF4* gene dosage and protein function, in contrast e.g., to other neuronal bHLH factors, is particularly susceptible to interference. Thus, TCF4 availability for unknown homo- and/or heterodimeric bHLH complexes represents a critical bottleneck in neurodevelopmental processes that might be associated with an increased risk of schizophrenia. Reduced *TCF4* activity (haploinsufficiency) has been shown to cause severe mental retardation as observable in the PTHS and may also be associated with other autism-spectrum disorders in humans [134, 138]. More subtle gene dosage alterations are likely to be associated with schizophrenia and possibly also bipolar disease. Gene dosage sensitivity may not be as pronounced in rodents compared to humans as heterozygous null mutant mice display only subtle neurodevelopmental disturbances although for example a thorough behavioural analysis of these mice is still missing [151].

Given the enormous complexity of *TCF4* splice variants and biochemical properties of different PTHS associated mutations [10, 11], it is still possible that dominant-negative effects beyond dosage effects contribute to the severity of the neurodevelopmental disturbances in humans. In a transgenic mouse model (*Tcf4tg*) with slightly elevated expression of *Tcf4* in the forebrain and displaying cognitive and sensorimotor deficits were observed supporting the critical gene dosage sensitivity [112]. However, potentially dominant negative effects by the corresponding C-terminally tagged protein expressed in the transgenic animals cannot be formally excluded since C-terminal frame shift mutations have been

shown to alter TCF4 functions [11]. Nonetheless, *Tcf4tg* mice display a dysbalance of *Neurod1* versus *Id2* expression ratios and it is thus plausible, that even slight disturbance of a delicate balance of bHLH transcription factor gene expression in the adult brain impairs cognition and information processing. In line with that, heterozygous *Neurod2* null mutants also display cognitive deficits [164]. Notably, the dominant HLH factors *Id2* and *Id4* display similar to *Tcf4*, *Neurod2* and *Neurod6* sustained expression in the adult brain indicating a dynamic control of adult *TCF4* function at the level of dimerization possibly coupled to neuronal activity possibly via nuclear Ca^{2+} signalling. It has been shown that TCF4 interacts with the Ca^{2+} binding protein calmodulin at physiological concentrations inhibiting DNA binding of E-protein homodimers in non-neuronal cells [165–169]. The mode of the Ca^{2+} mediated regulation of TCF4 function in neurons is not known but should, for several reasons, be of high interest for future attempts to understand the mechanisms of how TCF4 contributes to endophenotypes of schizophrenia. Firstly, localized Ca^{2+} signalling has been identified as a key player in communicating synaptic activity to the nucleus and to be critically involved in mediating transcription dependent adaptive responses in neurodevelopment, plasticity and cognitive processes [170, 171]. Secondly, recent cross-disorder analyses of GWAS data combined with pathway analysis provided strong evidence for the importance of L-type voltage gated Ca^{2+} channels (VGCC) and Ca^{2+} signalling in schizophrenia and bipolar disorders [172]. Most prominently, The intronic polymorphism rs4765914 in *CACNA1C* has been previously associated independently with bipolar disorder [173, 174], schizophrenia [19] and major depressive disorder [175]. Genetic imaging approaches linked *CACNA1C* variants along an endophenotypic spectrum similar to that observed for *TCF4* including attention deficits [176] and memory formation [177]. Of note, the C-terminus of *CACNA1C* encodes a transcription factor that is implicated in activity-transcription coupling by regulated proteolysis at the membrane [178]. Moreover, several other Ca^{2+} regulated transcription factors (CREB1, MECP2, MEF2, FOSB, NPAS4, CREST among others) have been associated with psychiatric diseases such as Rett-Syndrome, autism and bipolar disorder [170, 171, 179]. In addition, the validated TCF4 interaction partners NEUROD1 and NEUROD2 are themselves regulated by Ca^{2+} [179–182]. Although there is no direct experimental evidence for a particular mechanism by which synaptic activity/ Ca^{2+} could modulate TCF4 activity in neurons, several non-exclusive modes of action

are possible that are based on studies with different class I and II bHLH factors in non-neuronal cells (see above): (i) Regulation of transcription or splicing of *TCF4* or its interaction partners by Ca^{2+} regulated transcription or splicing factors, (ii) Ca^{2+} controlled cytoplasm to nucleus shuttling of *TCF4*, (iii) modulation of dimerization selectivity and DNA binding efficiency or specificity by either interaction with Ca^{2+} binding proteins such as calmodulin or posttranslational modifications via Ca^{2+} regulated kinases, which could also alter (iv) the recruitment of transcriptional co-factors such as p300/CBP, (v) transcriptional regulation of gene products involved in Ca^{2+} signalling.

The importance of a tightly controlled gene expression program in the context of schizophrenia is further supported by the findings that *mirR-137* is also a genetic risk factor and has been shown to target 3' regions in the human mRNA of *TCF4* [22]. Due to the promiscuity of micro RNAs, it is not surprising that *mirR-137* most likely regulates abundance levels of several mRNAs among which, however, may be a substantial fraction of schizophrenia risk associated gene products [183]. Similar to *TCF4*, *mirR-137* has been shown to be involved in the regulation of neuron maturation [184] and adult neurogenesis [185]. Most recently, a post-mortem study demonstrated that a decreased *mirR-137* expression – caused by the TT genotype of the SNP rs1625579 – was associated with increased *TCF4* expression [154]. Although the identification of putative *mirR-137* targets is mainly based on *in silico* predictions and has only partially been validated by reporter gene assays, it is possible that *mirR-137* also represents another 'hub' within gene regulatory programs that are considered to be of particular relevance for schizophrenia. Among the putative *mirR-137* targets beyond *TCF4* are several high confidence schizophrenia risk genes (*CSMD1*, *C10orf26*, *CACNA1C*, and *ZNF804A*) and members of schizophrenia-associated glutamatergic, GABAergic, serotonergic, and neuregulin-ErbB signaling pathways (*GRIN2A*, *GRM5*, *GABRA1*, *HTR2C*, *NRG2*, *NRG3* *ERBB4*) [183]. Although direct target genes of *TCF4* in the brain are not known and putative *mirR-137* targets have not been validated *in vivo*, growing evidence suggests that both factors are crucial players of gene expression networks that may be particularly susceptible to interference by environmental factors. In schizophrenia, multiple genes are thought to cooperate with different environmental factors in unfavourable combinations. Thus, future research should be dedicated to the elucidation of *TCF4* and *mirR-137* controlled gene regulatory

networks that may allow the elucidation of causal gene*interactions underlying schizophrenia symptoms.

It has been proposed that the inter-individual phenotypic variability and severity of PTHS may reflect the molecularly divergent mutations that compromise *TCF4* function differentially [11]. Similarly, we hypothesize that a graded level of ‘severity’ of *TCF4* dysfunction ranging from haploinsufficiency caused by missense mutations in PTHS [134, 135], to chromosomal aberrations [138], and subtle alterations induced by common genetic variants [19, 76, 77] correlates with the ‘severity’ of the corresponding neurodevelopmental diseases such as mental retardation, autism-spectrum disorders and schizophrenia. The most obvious common feature of these diseases is the graded intellectual and cognitive impairment. As described above, accumulating data from both human and mouse studies suggests that *TCF4* dysfunction might be particularly important for higher order cognitive processing. Therefore, it may be possible that overlapping mechanisms and/or pathways are affected by *TCF4* which could have implications for the focus of future experiments. Assuming that similar mechanisms are rather quantitatively altered e.g., in PTHS and schizophrenia (and not categorically qualitatively different), the identification and validation of *TCF4* target genes in PTHS and corresponding loss-of-function mouse models could well be of relevance for schizophrenia. The genetic complexity of schizophrenia *per se* and the subtle alterations at the gene expression level that one has to assume to occur from the schizophrenia-associated non-coding *TCF4* variants obviously hampers the identification of target genes from patient derived samples or schizophrenia mouse models. Therefore, the fact that different genetic alterations in *TCF4* are causally associated with several phenotypically overlapping mental disorders could help to guide experimentally feasible attempts to obtain further mechanistic insights in the function of this gene. Future studies on *TCF4* should thus not strictly focus exclusively on models with construct-validity for schizophrenia, which may be out of reach at the moment, but should (as the different types of mutations in the gene) step beyond disorder boundaries by e.g., analysing genetically defined cellular and animal gain- and loss-of-function models in more depth. In addition, observations from human studies could foster translational studies in model systems approaching gene*environment interactions with relevance for schizophrenia (see below).

By combining electrophysiological measurements with genetics it has been shown that *TCF4* risk alleles correlate with particular schizophrenia endophenotypes – namely sensory and sensorimotor gating [112–114]. Specifically sensory gating revealed an interesting and unexpected gene*environment interaction: The schizophrenia risk allele C of the *TCF4* rs9960767 SNP was robustly associated with reduced P50 suppression of the AEP. However, this genotype effect was strongly modulated by smoking behaviour given that only smokers showed reliable *TCF4*-sensory gating associations, while the gene effect was not present in never-smokers. Moreover, the genotype*smoking interaction was dose-related as the *TCF4* genotype effect grows with increasing smoking severity [114]. However, the moderating influence of smoking on the *TCF4* genotype effect was not present in the previous investigation on *TCF4* gene effects on sensorimotor gating measured by PPI [113]. The earlier investigated samples might have been too small and underpowered (healthy sample: N=98; schizophrenia spectrum sample: N=105) to reliably examine the effects of smoking as a mediating factor on the *TCF4* gene effects on PPI. The potentially moderating effect of smoking on *TCF4* gene effects on PPI (and other schizophrenia endophenotypes) should therefore be investigated in larger samples. Finally, the *TCF4* genotype effect on PPI displayed a much stronger effect size (Cohen's $d=0.90$) than the mean effect on P50 suppression (mean $d=0.23$, ranging from 0.03 in never-smokers to 0.69 in heavy smokers), which could partially be explained by a superior reliability of PPI compared to P50 suppression [114].

But how could the unexpected smoking*genotype interaction regarding P50 suppression be elucidated? There are at least two possible explanations: The first is a hidden gene*gene interaction: In this model *TCF4* interacts with a hidden gene (or genes) so that only the presence of two or more risk alleles is associated with both smoking severity and P50 suppression, while *TCF4* alone was merely associated with P50 suppression but not with smoking. Further studies might investigate possible gene*gene interactions and promising candidates for the “hidden” SNPs may lay in the *CHRNA3-CHRNA5-CHRNA4* gene cluster coding for $\alpha 3$, $\alpha 5$, and $\beta 4$ nicotinic acetylcholine receptor (nAChR) subtypes. SNPs from this gene cluster were reliably associated with smoking behaviour [186–190], and also with sensorimotor gating (PPI) [191] and cognitive performance [192]. The second and

maybe more appealing explanation for the present result pattern could be a gene*environment interaction, in which smoking represents a long-lasting and ongoing environmental influence. This interpretation would be in line with the suggestion of Williams et al [41] that the *TCF4* schizophrenia risk allele may exert its effect on expression exclusively in a developmental context because their post-mortem data suggested that this SNP is neither functional nor affects mRNA expression in the adult human brain. However, at the moment we can only hypothesize, which neurobiological mechanisms might underlie this *TCF4**smoking interaction on P50 suppression. Using *TCF4* knock-out mice it was recently shown that *TCF4* plays a unique role in the development of the pontine nuclei [151]. These nuclei are highly interconnected with the cochlear nucleus and neighboring brain stem nuclei that are critically involved in auditory information processing [193, 194]. Moreover, pontine nuclei are also connected to the pedunculopontine nucleus [195], which has been shown to be critically involved in auditory sensory gating and sensorimotor gating in animal studies [196–199]. Most of the auditory pathways within the brain stem are mediated by cholinergic neurotransmission and the predominant nAChR expressed in the lower auditory brainstem nuclei is the $\alpha 7$ subtype, while $\alpha 3\beta 4$ nAChR also plays a role but rather in the development of the auditory brainstem system [200]. Given that repeated exposure to nicotine results in nAChR desensitization [201, 202] and also to a long-term homeostatic increase of $\alpha 4\beta 2$ and $\alpha 7$ nAChR [203, 204], smoking-induced changes in brainstem nAChR function might interact with developmental changes within pontine nuclei resulting in changes of P50 suppression. Actually, P50 amplitude to S1 was influenced by smoking but not by *TCF4* genotype and, therefore, basic auditory processing was somewhat affected by smoking but not directly influenced by *TCF4*. Moreover, changes in nAChR function induced by chronic nicotine exposure might impact auditory sensory gating also at neocortical or hippocampal levels [205]. Taken together, smoking-induced plasticity of nAChR in concert with neurodevelopmental changes induced by *TCF4* gene variations may have affected P50 suppression in our sample. Alternatively, nicotine may be involved in the methylation of DNA sequences within the *TCF4* gene or other genes interacting with *TCF4* leading to an epigenetic change of the expression of the corresponding genes. It was previously shown that nicotine could decrease glutamic acid decarboxylase-67 and DNA methyltransferase-1 via epigenetic mechanisms, which are induced by an activation of nAChRs

located on cortical and hippocampal GABAergic interneurons [206]. Additionally, it has been recently demonstrated that smoking affects monoaminooxidase-A (*MAOA*) promoter methylation in DNA prepared from lymphoblasts and whole blood [207]. Interestingly, quitting smoking did not lead to a return to methylation levels found in never-smokers indicating a long-lasting effect of smoking on DNA methylation. Thus, smoking might exert a sustained impact on central *MAOA* activity (and other genes) via epigenetic mechanisms leading to changes in noradrenergic function that interact with neurodevelopmental changes caused by *TCF4* gene variations (see above). Eventually, nicotine might impact the expression of *TCF4* gene also directly with functional consequences on early information processing. In summary, the unexpected findings in humans of *TCF4**smoking interactions inspired the formulation of several hypotheses linking different biological systems as additional modulators of *TCF4*-associated endophenotypes i.e. information processing and cognition. These, in turn, could be further investigated in *Tcf4tg* mice which display complementary endophenotypes and may offer predictive value for validation and possible pre-clinical studies [112, 119]. Ameliorating the cognitive impairments in schizophrenia, most likely caused by higher order information processing deficits in dispersed brain circuits, still represents a critical unmet medical need to finally improve the therapeutic options for most schizophrenic patients. The elucidation of dedicated *TCF4* risk variant-associated endophenotypes and corresponding molecular mechanisms certainly represent first steps towards this goal.

Conclusions

TCF4 is still one of the most promising schizophrenia risk genes as it is slightly but replicable associated with the illness, it is more strongly related to gating endophenotypes of schizophrenia, and it seems to be susceptible for environmental impact as discussed above. Moreover, it obviously plays an important role in brain development and is connected to the function of other genes such as *miR-137*, which are also discussed as schizophrenia risk genes. Taken together, a causal role of *TCF4* for schizophrenia would be in line with neurodevelopmental hypotheses as well as with repeated-hit models and G*E interaction models. Thus, *TCF4* as a schizophrenia risk gene would be versatile model that has the potential to integrate several schizophrenia models previously suggested. However,

the small gene effects in large schizophrenia patient populations and the stronger gene effects regarding gating endophenotypes might indicate that there is a subgroup of patients, in which *TCF4* plays a major role during pathogenesis, while most of the patients have different pathogenic pathways. Thus, future research might accomplish the identification of a specific *TCF4*-lead schizophrenia by combining genotyping and (endo)phenotyping. Finally, it is also conceivable that a combination of risk genes ranging from the *TCF4* associated bHLH system including interaction partners and target genes as well as associated regulatory mechanisms such as *miR-137* and possibly Ca^{2+} linked signalling networks, could represent a '*TCF4* gene-set' regulating neuronal growth and differentiation in a highly redundant network. These genes might cooperatively be responsible for the pathogenesis within a subgroup of schizophrenia patients. Due to the high functional redundancy in this network a critical mass of genetically and environmentally induced dysfunction is needed before the systems breaks down. Thus, we might focus on gene sets within the bHLH system and neighbouring regulatory systems to identify patients with a strong genetic and developmental pathogenesis. A postulated *TCF4* associated network of risk factors might potentially be suitable as an early indicator for a schizophrenic subtype. When combined with electrophysiological gating measures (such as PPI or P50 suppression), smoking behaviour and cognitive performance, corresponding molecular profiling could guide future stratified sub-population directed therapies.

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Figure Legends

Fig. 1 Different bHLH transcription factors direct central nervous system (CNS) development at embryonic stages and may be involved in adult brain plasticity. Inhibitory bHLH factors (HES1, ID1) and proneural factors ATOH1, ASCL1 and NEUROG1,2 as well as E-proteins TCF3 and TCF12 are involved in early developmental stages. The temporal expression patterns and mutational analyses of the neurogenic differentiation factors (NEUROD1,2 and 6) and inhibitors of differentiation ID2 and ID4 suggest rather a function in later stages of neuronal differentiation and in the adult CNS. The spatiotemporal expression pattern of *Tcf4* overlaps substantially with all other bHLH factors involved in brain development. Moreover, TCF4 is capable of forming hetero-dimers with most involved neuron expressed bHLH factors although direct evidence is thus far only available for NEUROD1 and -2 (as indicated by a solid in contrast to dashed lines). It should be noted that this schematic drawing is thought to be an overview representation not claiming detailed spatial and temporal expression domains of single genes (for citations see main text).

Fig.2 Phenotypical comparisons reveal different *TCF4* gene dosage dependences in mice (A) and humans (B) in neurodevelopment related diseases including schizophrenia. Gain-of-function and loss-of-function analyses in mice and corresponding risk alleles and mutations in humans suggest that *TCF4* expression differences are tolerated in a narrow range (range depicted in light blue). Exceeding critical thresholds increases disease risks (depicted in grey). Slightly increased postnatal expression of *Tcf4* has been found to cause schizophrenia (SZ) associated symptoms in mice. Phenotypic consequences of increased *Tcf4* expression during embryonal stages are not known yet (A). Indirect evidence from human post-mortem brain and blood sampling suggests that elevated expression may be associated with SZ and bipolar disease (BD). The critical period of enhanced *TCF4* expression in humans is unknown (B). The tolerance range for reduced gene dosage effects might potentially be higher in mice compared to humans since heterozygous animals appear to be largely unaffected although a thorough behavioral phenotyping has not been performed so far. Thus, it is unknown if reduced gene dosage in mice may cause SZ-like symptoms. The analysis of null mutants is hampered by perinatal lethality but structural deficits in brain development have been described already although

not representing Pitt-Hopkins like symptoms thus far (A). Loss-of-function of *TCF4* (haploinsufficiency and mosaic deficiency) causes severe neurodevelopmental diseases including PTHS and possibly other autism-like syndromes. Given many examples of inverted-U-shape relationships of gene dosage with disease severity in autism-related neurodevelopmental diseases, it appears possible that slightly reduced expression levels of *TCF4* may be implicated in SZ (B) (for citations see main text). SZ, schizophrenia; BD, bipolar disorder; PTHS, Pitt-Hopkins Syndrome; NDD, neurodevelopmental disorder; MR, mental retardation.

Fig. 3 Brain structures involved in postulated deficits of information processing in mice and men. Behavioural and neuropsychological phenotypes obtained in mice (A) and human subjects (B) suggest a function of *TCF4* in brain networks that are important for cognition (bold lines) and sensory processing (dotted grey lines). Deregulation of *TCF4* expression levels during development interferes with proper functional connectivity within corresponding brain networks (for citations see main text). ACx, auditory cortex; Amy, amygdala; Hi, hippocampus; NAc, nucleus accumbens; PCx, prefrontal cortex; PPN, pedunculo pontine nucleus; VTA, ventral tegmental area; VP, ventral pallidum;

Table 1: Single nucleotide polymorphisms of the transcription factor 4 (TCF4) associated with schizophrenia and schizophrenia endophenotypes

Phenotype / endophenotype	TCF4 SNPs	Significant association with minor allele	Study type and samples	Ethnicity	References
Schizophrenia	rs9960767	OR ^c = 1.20-1.23	GWAS ^a with partially overlapping samples: 12945 to 18206 SZ patients 34591 to 42536 controls	European ancestry (also including European-Americans and European - Australians)	Stefansson et al. 2009 [76]
	rs17512836	OR = 1.23			Ripke et al. 2011 [19]
	rs4309482	OR = 1.09			Steinberg et al. 2011 [77]
Schizophrenia	rs2958182	OR = 0.78	Single association study: 2496 SZ patients 5184 controls	Han Chinese	Li et al. 2010 [78]
Schizophrenia	rs9960767	OR = 1.18	GWAS: 1606 SZ patients 1794 controls	Irish	Strange et al. (2012) [79]
	rs17594526 ^d	OR = 1.77			
Schizophrenia	rs1261117	OR = 1.6	Family-based linkage meta-analysis: 6298 individuals (including 3286 SZ patients) from 1811 nuclear families	European ancestry	Aberg et al. 2013 [80]
Multivariate schizophrenia phenotype including positive, negative, and cognitive symptoms, neurological soft signs, and age of prodromal onset	rs9960767	Risk allele C was associated with more pronounced schizophrenia phenotype (only trend, not surviving correction for multiple testing)	Phenotype-based association study: 1041 SZ patients 1144 controls	German	Papiol et al. 2011 [82]
Antipsychotic drug response in SZ patients	rs9960767	No effect	Pharmacogenetic association study: 214 SZ patients in total (two independent samples with N=70 and N=144 SZ patients)	German	Lennertz et al. 2011 [84]

Prepulse inhibition of the acoustic startle response (sensorimotor gating)	rs9960767	Risk allele was associated with schizophrenia-like endophenotype OR ^e = 6.82 (SZ) OR ^e = 4.93 (controls) OR ^e = 4.81 (total sample)	Endophenotype-based association study in two independent samples: 105 SZ patients and high risk subjects 98 controls	SZ patients: German Controls: British	Quednow et al. 2011 [113]
P50 suppression of the auditory evoked potential (sensory gating)	rs9960767 rs17512836 rs17597926 ^d rs10401120	Risk alleles were associated with schizophrenia-like endophenotype OR ^e = 1.23-1.46 (never-smokers) OR ^e = 2.10-2.44 (light smokers) OR ^e = 3.21-5.50 (heavy smokers) OR ^e = 1.81-1.94 (total sample)	Endophenotype-based association study: 1821 controls	German	Quednow et al. 2012 [114]
Word recognition	rs9960767	Risk allele C was associated with enhanced performance	Endophenotype-based association study: 401 SZ patients	German	Lennertz et al. 2011 [127]
Attention and vigilance Working memory Processing speed Visuo-motor speed/set-shifting Verbal fluency	rs9960767	No effect	Endophenotype-based association study: 198 SZ patients 205 controls	German	Lennertz et al. 2011 [84]
Verbal fluency	rs12966547 rs4309482	Risk alleles were associated with	Endophenotype-based association study: 596 psychotic patients (including patients	Norwegian	Wirgenes et al. 2012 [83]

		poor performance	with SZ, bipolar disorder, or other psychoses) 385 controls		
Intelligence Test (WAIS-RC ^f) Several attention-related tasks	rs2958182	Risk allele was associated with better performance in patients but worse in controls	Endophenotype-based association study: 580 SZ patients 498 controls	Han Chinese	Zhu et al. 2013 [128]
Reasoning and problem solving Processing speed	rs9960767	Risk allele was associated with lower performance	Endophenotype-based association study: 173 first-episode psychosis patients	Canadian	Albanna et al. 2013 [129]

^aGWAS: Genome-wide association study

^bSZ: Schizophrenia

^cOR: Odds ratio

^dThis SNP was also among the top ten significant *TCF4* SNPs in the so far largest megal-GWAS-analysis of Ripke et al. (2011) [19].

^eCriterion one standard deviation from the control population

^fWechsler Adult Intelligence Scale-Revised

Figure 1

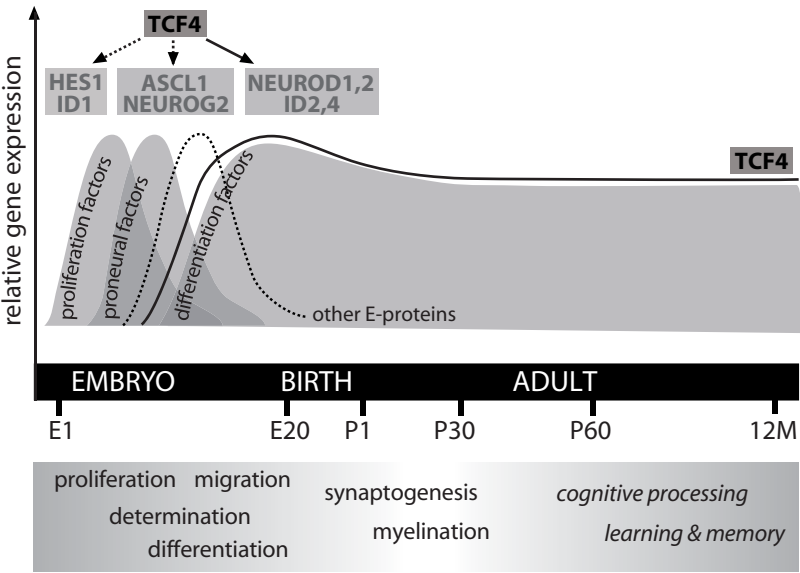


Figure 2

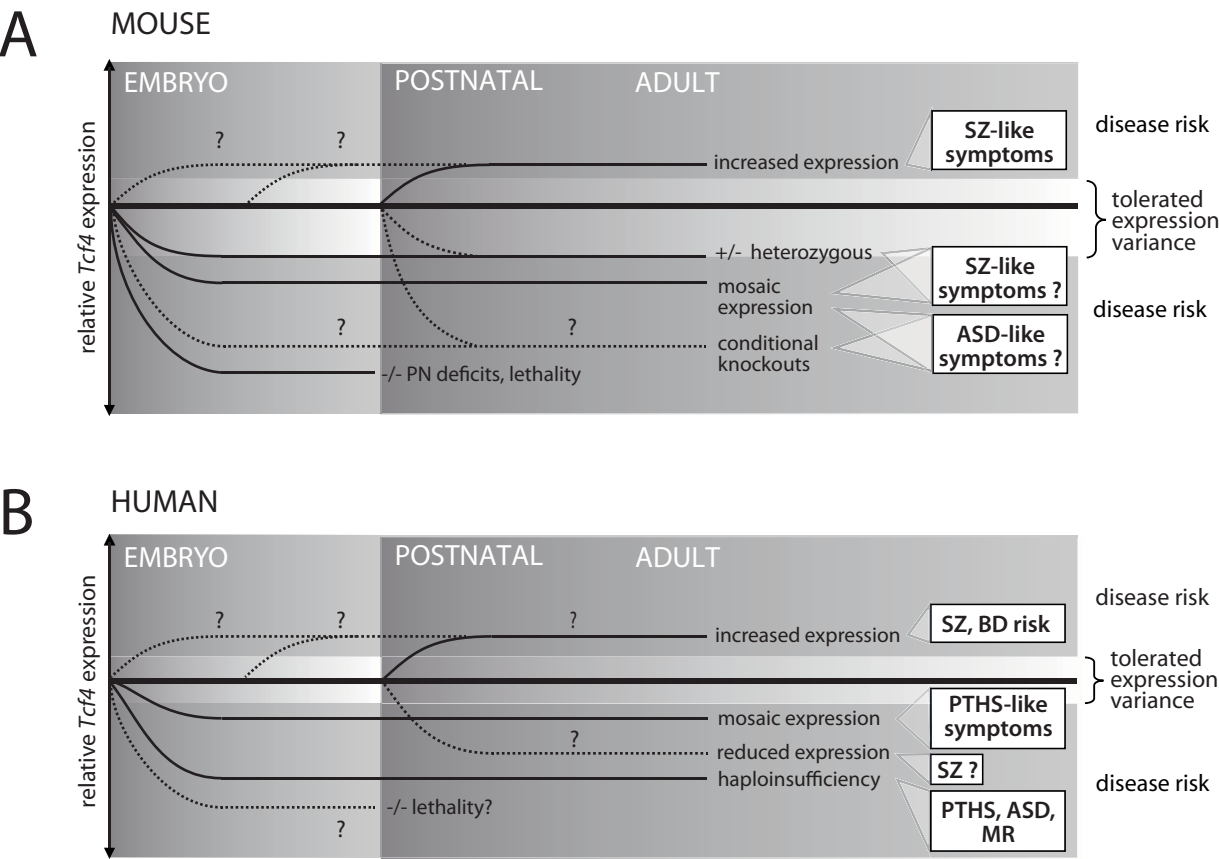


Figure 3

